UNIVERSITI PUTRA MALAYSIA

THE ISOLATION AND PARTIAL CHARACTERISATION OF THE CHALCONE SYNTHASE, FLAVANONE 3-HYDROXYLASE AND PHYTOENE SYNTHASE GENE FRAGMENTS FROM *ONCIDIUM TAKA* BY USING RT-PCR

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The RT-PCR technique was used to isolate partial gene fragments for the chalcone synthase (CHS), flavanone 3-hydroxylase (F3H) and phytoene synthase (PSY) genes from *O. taka*. The RT-PCR products were amplified by degenerate primers specifically designed for the genes and the templates (total RNA) were prepared from the leaves, open flowers and flower buds of *O. taka*. A 650bp RT-PCR product was successfully amplified from the three different total RNA templates when the degenerate primers for CHS were used. Similarly, a 543bp RT-PCR product was obtained when the degenerate primers for PSY were used on the three different total RNA templates. Only the total RNA preparation from flower buds gave a 503bp RT-PCR product when the degenerate primers for F3H were used.

The deduced amino acid sequence for the 650bp DNA fragment was found to have a high homology to other CHS sequences in the Genebank, averaging at 66%. Additionally, this sequence also has an exceptionally high homology to previously reported bibenzyl synthase (BibSyl) sequences, with an average percentage of 85%. The 503bp fragment has on average 76% homology to the F3H sequences reported for other plant species. As for the 543bp fragment, it has an average of 76% homology to other PSY gene sequences published at Genebank. The results indicate that the CHS and PSY genes are expressed in all the tissues tested whereas the F3H gene is only expressed at the flower bud stage in *O. taka*.

These gene fragments were labelled with DIG and used as probes to screen a genomic DNA library constructed from partially digested genomic DNA of *O. taka*. Currently, the F3H probe (503bp DNA fragment) has led to the isolation of a genomic clone with an insert size of around 11kb. The genomic clone was restricted into 2 fragments with *Bam*H I and subcloned into the pUC18 vector separately. The characterisation of the clone is underway.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENCILAN DAN PENCIRIAN SEPARA SEBAHAGIAN GEN CHALCONE SYNTHASE, FLAVANONE 3-HYDROXYLASE DAN PHYTOENE SYNTHASE DARIPADA *ONCIDIUM TAKA* DENGAN TEKNIK RT-PCR

Oleh

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Pemencilan gen-gen separa untuk gen chalcone synthase (CHS), flavanone 3-hydroxylase (F3H) dan phytoene synthase (PSY) daripada *O. taka* dicapai dengan menggunakan teknik RT-PCR. Produk-produk RT-PCR yang diamplifikasikan oleh pencetus-pencetus degenerasi adalah dicipta khas untuk gen-gen tersebut. Templat 'total RNA' untuk RT-PCR diekstrak daripada daun, bunga dan kudup bunga *O. taka*. Dengan cara ini, RT-PCR produk yang bersaiz 650bp telah berjaya diamplifikasikan daripada ketiga-tiga jenis total RNA templat apabila pencetus degenerasi untuk CHS digunakan. RT-PCR produk juga berjaya diamplifikasikan daripada ketiga-tiga jenis total RNA templat apabila pencetus degenerasi untuk PSY digunakan dan saiz produk tersebut ialah 543bp. Bagaimanapun, hanya templat total RNA yang diekstrak daripada kudup bunga yang dapat memberi satu produk yang bersaiz 503bp apabila pencetus degenerasi digunakan.

Jujukan asid amino untuk fragmen DNA 650bp didapati mempunyai persamaan sebanyak 66% secara purata dengan jujukan-jujukan asid amino CHS yang terdapat di Genebank. Selain daripada itu, jujukan asid amino tersebut juga mempunyai persamaan yang tinggi kepada jujukan-jujukan gen bibenzyl synthase (BibSyl), iaitu sebanyak 85%. Untuk fragmen DNA yang bersaiz 503bp, jujukan asid aminonya mempunyai purata persamaan sebanyak 76% kepada jujukan-jujukan asid amino F3H yang dilaporkan untuk species tumbuhan yang lain. Untuk fragmen DNA yang bersaiz 543bp pula, jujukan asid aminonya mempunyai purata persamaan sebanyak 76% dengan jujukan asid amino PSY yang telah diterbitkan dalam Genebank. Keputusan di atas memberi makna bahawa gen-gen CHS dan PSY diekspreskan di dalam semua tisu yang dikaji dan gen F3H hanya diekpreskan pada tahap kudup bunga di dalam *O. taka.*

Gen-gen fragmen telah dilabel dengan DIG dan digunakan sebagai prob untuk menyaringi khazanah DNA genomik yang disediakan daripada genomik DNA *O. taka* yang dipotong separa oleh enzim *Mbo* I. Pada masa kini, prob F3H (fragmen DNA 503bp) berjaya memencilkan satu klon genomik yang mempunyai saiz 'insert' lebih kurang 11kb. Klon genomik tersebut telah dipotong oleh enzim BamH I kepada dua fragmen dan diklon ke dalam vektor pUC18. Kerja pencirian klon-klon tersebut sedang dijalankan.

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LIST OF ABBREVIATIONS

cDNA	-	complementary deoxyribonucleic acid
CTAB	-	cetyltriaminebromide
DNA	-	deoxyribonucleic acid
DIG	-	digoxigenin
E. coli	-	*Escherichia coli*
EDTA	-	ethylenediamine tetraacetate
EtBr	-	ethidium bromide
LB	-	Luria Bertani
M	-	molarity
mL	-	mililitre
mRNA	-	messenger ribonucleic acid
O.D	-	optical density
RE	-	restriction enzyme
RT-PCR	-	Reverse Transcriptase-Ploymerase Chain Reaction
RNA	-	ribonucleic acid
X-Gal	-	5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase

CHAPTER I

INTRODUCTION

Flower colouration is facilitated by the existence of coloured compounds such as flavonoids, carotenoids and betalains. Between flavonoids and carotenoid, the former is the major contributor to floral pigmentation. These universally available compounds in the plant kingdom are found in the vacuoles of the cells of vascular plants. The variety of anthocyanins in higher plants confers a wide spectrum of colours. For example, pelargonidins usually give rise to orange colours, cyanidins to red colours and delphinidins to purple colours. Carotenoids are yellow coloured compounds that can impart colours in the range of reddish to yellowish hues .

The production of flavonoids is systematically controlled by a set of structural and regulatory genes in the flavonoid biosynthesis pathway. The biochemistry and genetics for this pathway has been well studied over the past decades in species such as maize (Dooner, 1982), petunia (de Vlaming, *et al.*, 1984) and *Antirrhinum* (Martin, 1987). Many structural and regulatory genes have been identified and isolated from these species. These studies have specifically led to the identification of the major structural genes that were involved in the flavonoid biosynthesis

pathway, namely chlacone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H) and many others. In orchids however, there has been little study of this pathway until recently (Liew *et al.*, 1995 and Hsu *et al.*, 1997). Furthermore, the phytoene synthase gene, which is instrumental in the development of the compound carotenoids, has never been identified in orchid flowers although carotenoids has been known to be the major compound found in the flowers of tomato.

The diversity of the Orchidaceae family may offer a new perspective in flower colour formation and regulation, as its genetic and morphological make up is very different from other species. Obviously, the isolation of the genes that encode the enzymes that synthesise flavonoids and their regulatory counterparts will provide a better understanding to the flavonoid biosynthesis mechanism in this family.

In this study, the orchid *Oncidium sp.* is of particular interest and this stems from the fact the majority of these species only produces yellow colour flowers. Crossbreeding has little success in obtaining truly white phenotypes. The isolation and characterisation of the genes in the flavonoid biosynthesis pathway and the carotenoid biosynthetic pathway in this species will provide a backbone for understanding the colour formation. With this knowledge, flower colour manipulation for orchids will be a reality and eventually be commercially beneficial.

Objectives

The objective of this study is to isolate the partial gene fragment of chalcone synthase (CHS), flavanone 3-hydroxylase (F3H) and phytoene synthase (PSY) by Reverse Trancribed-Polymerase Chain Reaction (RT-PCR). Additionally , the isolated gene fragments will be partially characterised.

CHAPTER II

LITERATURE REVIEW

The Flavonoid Biosynthetic Pathway

The condensation of three acetate units and one hydroxycinnamic acid unit is the basic carbon skeleton of the flavonoid molecule. This product, chalcone is a central intermediate in flavonoid biosynthesis (Birch and Donovan, 1953). Flavonoids such as flavones, flavonols and anthocyanins are produced from the modifications of chalcones. Even though flavones and flavonols do affect pigmentation indirectly, it is the anthocyanins that contribute to flower colouration directly.

The route to the production of anthocyanins involved a series of enzymes in flavonoid biosynthetic pathway (Figure 1). Phenylalanine ammonia lyase (PAL), being an enzyme that belongs to the phenylpropanoid metabolism pathway catalyses the transelimination of ammonia from phenylalanine to form *trans*-cinnamate (Hanson and Havir, 1972; 1981). Further modifications give rise to 4-coumaroyl CoA, which is the first substrate for the flavonoid biosynthetic pathway. The condensation of 4-coumaroyl CoA with three molecules of malonyl CoA by the first enzyme of the pathway, chalcone synthase (CHS) gives rise to chalcone (Heller and Hahlbrock, 1980). The subsequent step involves the closing of the stereo-specific ring giving rise to flavanone (Hahlbrock and Grisebach, 1970) and this reaction is

performed by chalcone isomerase (CHI). The next enzymatic reaction converts flavanones to dihydroflavanols through hydroxylation (Fritsch and Grisebach, 1975). Dihydroflavonol serves as direct precursors for anthocyanin synthesis. The reduction at the fourth position of the C ring produce a compound called leucoanthocyanidin and this step is the responsibility of an enzyme named dihydroflavonol 4-reductase (DFR) (Stafford and Lester, 1982).

The first coloured compound emerges when leucoanthocyanidins are converted to anthocyanidins by an uncharacterised enzyme through the actions of hydroxylation and dehydration (Heller and Forkmann, 1988). Consequently, the addition of a glucose residue at the third position of the C ring of the anthocyanidin by UDPG: flavonoid 3-o-glucosyltransferase (UFGT) stabilises the aglycones (Larson and Coe, 1968).

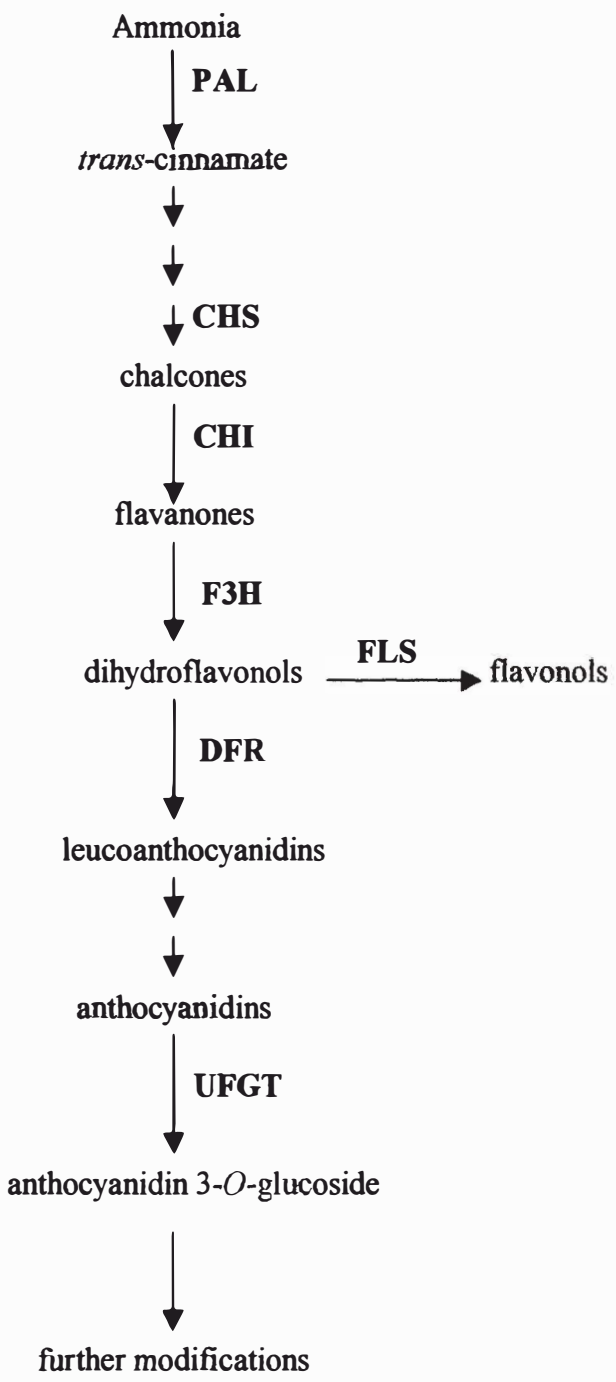

Figure 1: A schematic representation of the flavonoid biosynthetic pathway.
PAL, phenylalanine ammonia lyase
CHS, chalcone synthase
CHI, chalcone isomerase
F3H, flavanone 3-hydroxylase
FLS, flavonol synthase
DFR, dihydroflavonol 4-reductase
UFGT, UDPG: flavonoid 3-o-glucosyltransferase

Characteristics of Chalcone Synthase

The first committed step in the flavonoid biosynthetic pathway is performed by chalcone synthase (CHS). It catalyzes the condensation of three molecules of malonyl CoA and one of p-coumaroyl to produce chalcone (Heller and Hahlbrock, 1980).

The synthesis of CHS is known to be tightly regulated. The CHS gene is highly expressed in the early development of most plant tissues and eventually its presence will only be detected in a few tissues of adult plants (Kreuzaler, 1974). This suggests that the CHS gene developmentally regulated. Apart from this, this enzyme can also be induced by the phytochrome system, UV-light (Kreuzaler *et al*., 1981) or elicitors (Lawton *et al*., 1983). A good demonstration of elicitors inducing the action of CHS is found in the inoculation of the non-pathogenic fungus *Cochliobolus heterostrophus* into *Sorghum bicolor* L. Moench (Lo and Hicholson, 1998). This inoculation was found to significantly reduce the light-induced accumulation of anthocyanin by repressing the transcription of F3H, DFR and anthocyanidin synthase. However, this infection resulted in the synthesis of four genes in the phytoalexin biosynthesis pathway, a corresponding activation of the genes encoding the key branch-point enzymes in the phenylpropanoid pathway, phenylalanine ammonia-lyase and CHS.

The CHS gene is also known to play an important role in anther development. In anthers, CHS is located in the tapetal cells (Kehrel and Wiermann, 1985). In additon to that, a clone BA42 which is found to encode a protein sharing 64-67% similarity

to CHS (Jeanie and Francis, 1992) is expressed in tapetum, periphery of vascular bundle and microspore of immature anther of *Brassica napus*.

In *Antirrhinum majus* the chalcone synthase gene are shown to be encoded by the *nivea* locus (Spribille and Forkman, 1982). In maize, the CHS gene is encoded by the *c2* gene and it is expressed in the aleurone layer of the seed (Dooner, 1983). Another locus that codes for the CHS gene in maize is *Whp* (Coe *et al*., 1981) and is responsible for depositing flavonoids in pollen grains to facilitate normal pollen function. Although most CHS genes that have been isolated so far are single copy genes, there have been cases where a group of closely related genes code for the CHS gene. For example, there are twelve CHS homologous genes present in the petunia genome even though only two are expressed at significant levels (Koes and Spelt, 1989). In soybean, *Glycine max* (L) Merr., the CHS gene contains six family members (Wingender *et al*., 1989).

Since CHS is one of the key enzymes in the flavonoid biosynthetic pathway, much research has been carried out to determine which gene was either down regulated or up regulated. For example, the introduction of an antisense construct that consisted of the CHS gene caused a dramatic inhibition of the CHS gene expression in *Petunia* that resulted in white colour flowers (van der Krol *et al*., 1988, 1990a, 1990b). In another similar experiment, aberrant pollen development or germination was observed in CHS-antisense *Petunia* plants (van der Meer, *et al*., 1992).